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Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review

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ABSTRACT

Protocols for the cryopreservation of fish gametes have been developed for many different fish species, in special, freshwater salmonids and cyprinids. Methods for sperm freezing have progressed during the last decades due to the increasing number of potential applications: aquaculture (genetic improvement programs, broodstock management, helping with species having reproductive problems), biotechnology studies using model fish species (preservation of transgenic or mutant lines), cryobanking of genetic resources from endangered species, etc.

This mini-review tries to give an overview of the present situation of this area of research, identifying the main challenges and perspectives, redirecting the reader to more in-depth reviews and papers.

1. Progress

1.1. Applications of fish gamete cryopreservation

Cryopreservation of fish gametes has evolved during the last decades due to the increasing number of potential applications. The most evident is its use for aquaculture purposes, allowing the improvement of broodstock management at hatcheries (for example, modifying the offspring production season), preserving the genetically selected strains resulting from genetic improvement programs, or helping with species having reproductive problems as lack of synchronization in the gamete production of male and females (as in the case of the European eel, *Anguilla anguilla*; Asturiano et al., 2004) or with those having a low sperm production (as in the case of F1 Senegalese sole, *Solea senegalensis*; Cabrita et al., 2006).

Another potential application is the preservation of genetic material from individuals of natural populations of fish species in the initial phases of the domestication process and genetic modifications. This can assist in maintaining the original wild genotypes for the recovery of genes in the future (becoming a phenotypic backup), contrarily with happened for example in the case of the domestication process of bovine cattle (Vandeputte, 2011). Other possible conservation-related uses include the storage of genetic resources of the increasing number of fish in the lists of endangered species, allowing cryobanking for biodiversity (Van Der Walt et al., 1993; Martínez-Páramo et al., 2009, in press), or in the case of fish species recently attracting the interest of cryobiologists and aquaculturists, mainly in South America and Asia (Viveiros and Godinho, 2009).

Moreover, the increasing use of aquatic models such as zebrafish in studies of biotechnology, toxicology or pharmacology, requires the use of transgenic lines, knockout and mutant strains that need adequate storage (Kollár et al., 2015; Tiersch et al., 2011).

1.2. Cryopreservation of fish sperm

Fish genome cryobanking has been attempted using different cell types (see Section 2.3; Labbé et al., 2013). However, spermatozoa have been the objective of most of the studies, making sperm cryopreservation the most established and commercialized technique. The choice of this type of cell is because it is easy to collect in most of the fish species, has a simple cellular structure and a small size and high chilling resistance, making these cells easy to preserve in many fish species. Moreover, reconstruction

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of individuals can be done by normal fertilization (or androgenesis), but it allows the preservation of only male germplasm.

Some previous publications have reviewed fish sperm cryopreservation subject (Suquet et al., 2000; Cabrita et al., 2009a; Kopeika and Kopeika, 2008; Tiersch and Green, 2011; Figueroa et al., 2014). The Table 1 summarizes studies on cryopreservation of sperm from fish species published during the last 15 years, including the cryoprotectants used (and their concentrations) and the best results obtained in each case in terms of post-thaw motility, cell viability and fertilization rates.

2. Challenges

2.1. When biodiversity means problems

With 25,000–30,000 species, fish are the largest group of vertebrates, displaying an extreme biodiversity (Near et al., 2013). This biodiversity is evident in the significant differences found in gamete (spermatozoa) morphology and biology (Mattei, 1991). During the cryopreservation steps of cooling, freezing and thawing, some biophysical and chemical processes such as osmotic changes, dehydration and rehydration, cell volume changes, ice crystals formation, cryoprotectants toxicity, etc., occur and cells (gametes or others) are more or less sensitive to these changes being species-specific (Cabrita et al., 2014). Thus, cryopreservation protocols must be adapted to find species-specific compromises, and the increasing numbers of studies describing methods to cryopreserve sperm in many species, evidences this diversity (Cabrita et al., 2009a).

2.2. Lack of standardization: a problem to compare results and to arrive to the industry

The main objective has always been maintaining a high sperm fertilizing ability after thawing. However, the difficulties in obtaining reproducible results using sperm cryopreserved using the published methods have limited the use of cryopreserved sperm in production.

Recent scientific discussion have evidenced the need of standardization in different aspects of this area of research, as definition of basic concepts (extenders, cryoprotectant concentrations, dilution ratios), work protocols (sperm concentration determination, sperm cryopreservation methods, equilibration time, handling of straws, polystyrene box or controlled-rate freezer, type of vials, thawing systems, calculation of fertilization and hatching rates, osmolality measurements, sperm quality evaluation, etc) or even reporting of results (Rosenthal et al., 2010; Horváth et al., 2012a).

Although some recent efforts have been made in this regard (Benson et al., 2013; Gallego et al., 2012, 2013; Kása et al., 2014, 2015; Vílchez et al., 2014), new efforts must be made for a complete description and standardization of protocols for sperm cryopreservation, including a very wide area of topics: determination or estimation of sperm motility, substances used for activation of sperm, details of dilution of sperm with extender and cryoprotectants (new ones as the antifreeze proteins, AFPs, or better combinations of classic ones), use of straws (sealed or unsealed), cooling of samples (dry ice *vs*. liquid nitrogen, styrofoam box *vs*. programmable freezer), methods of calculating fertilization and hatching results.

Regardless of the very high number of publications on this topic, few of the published methods have been adapted to aquaculture practice. There can be several reasons for this failure of application; however, one of them is beyond doubt the lack of standardization not only in methodologies but also in reporting them correctly. The difficulties in interpretation and replication of methods lead to a disappointment and ultimately rejection by the aquaculture industry. We also need to understand that in most fish species sperm is not a limiting factor during induced spawning and, moreover, individual selection is not as advanced in fish as it is in terrestrial livestock.

2.3. Alternative cells

Fish genome cryobanking has been attempted using different cell types: spermatozoa, oocytes, spermatogonia and primordial germ cells (PGCs), as well as somatic cells, blastomeres and embryos (Labbé et al., 2013).

The cryopreservation of fish oocytes has severe limitations because of their large cell volume, the presence of a chorion, the low permeability to cryoprotectants, and a high chilling sensitivity. Different studies have been carried out in zebrafish, as well as other marine and freshwater species, including cryoprotectant toxicity, chilling sensitivity, membrane permeability and cryopreservation (cooling rates, vitrification) of oocytes at different stages of development or ovarian fragments (Zhang et al., 2007; Godoy et al., 2013; Streit Jr. et al., 2014; Marques et al., 2015; reviewed by Martínez-Páramo et al., in press). However, development of protocols for *in vitro* maturation of ovarian follicles after cryopreservation is required for the use of cryopreserved oocytes (Seki et al., 2008, 2011; Tsai et al., 2010). Thus, oocyte cryopreservation is still in its experimental phase and far from aquaculture applications.

The preservation of spermatogonia and primordial germ cell guarantees the full individual genome. These cells have been cryopreserved successfully in several fish species (Yoshizaki et al., 2011; Robles et al., in press). However, its use requires the development of specific biotechnological tools, such as transplantation.

Fish embryo cryopreservation could be perfect for the establishment and management of genetic selection programs in fish farms. However, they have low membrane permeability, low surface-to-volume ratio, large size, high yolk content and high chilling sensitivity (Hagedorn and Kleinhans, 2000), which is the primary reason for the very limited number of preliminary positive results (Chen and Tian, 2005; Martínez-Páramo et al., 2008; Robles et al., 2005).

The cryopreservation of somatic (diploid) or embryonic cells (including PGCs) is an alternative to the cryobanking of gametes. They can be a good source of diploid genome to reconstruct fish (reviewed by Labbé et al., 2013). Moreover, they can easily be collected (e.g.: fins clips that regenerate easily). However, the use of these cells means to develop a series of complex and specific techniques as cell culture, nuclear transfer or the transplantation of the thawed cells into recipient fish (of the same or related species) for individual restoration (Siripattarapravat et al., 2011; Chenais et al., 2014), that must be explored in different fish species (reviewed by Martínez-Páramo et al., in press).

2.4. Vitrification

This technique tries to prevent the negative effects of crystallization happening in the conventional cryopreservation methods mixing cryoprotectants at very high concentration and using very high freezing rates, getting the solidification of external and internal media into an amorphous/glassy state without formation of harmful ice crystals (Fahy et al., 1984).

First applications of vitrification to the cryopreservation of sperm of different species have been published (channel catfish, *Ictalurus* punctatus; Cuevas-Uribe et al., 2011a; green swordtail, *Xiphophorus hellerii*; Cuevas-Uribe et al., 2011b; rainbow trout, *Oncorhynchus mykiss*; Figueroa et al., 2013; Atlantic salmon, *Salmo salar*; Figueroa et al., 2015; Tambaqui, *Colossoma macropomum*; Varela Jr. et al., 2015; Eurasian perch, *Perca fluviatilis* and European